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Minireview

Attraction versus Repulsion: Modular Receptors Make the Difference in Axon Guidance

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Like so many fields of biology, progress in understanding the molecular mechanisms of axon guidance is proceeding at a frantic pace. During the last several years a multitude of proteins have been identified that play critical roles in guiding axons along their stereotypical pathways (see Tessier-Lavigne and Goodman, 1996 for a general review). Attractive and repulsive signals have been characterized, including the Ephrin, Netrin, Semaphorin, and the Slit protein families. Different classes of transmembrane receptors for these various guidance cues are also being identified and characterized. An unexpected theme is emerging for many of these guidance signals: several of these protein families contain members that act as bifunctional guidance cues, conferring both attractive and repulsive signals.

How does a growth cone respond to a bifunctional guidance cue? Are responses mediated by the repertoire of receptors on the growth cone surface, or are there other properties intrinsic to the growth cone that dictate the response? Two papers in this issue of *Cell* from the Goodman lab (Bashaw and Goodman, 1999) and from the Tessier-Lavigne and Poo labs (Hong et al., 1999) address this question. They find that transmembrane receptors determine whether a growth cone sees a cue as attractive or repulsive, consistent with earlier predictions. Moreover, the cytoplasmic domains of these receptors are the key effectors for initiating either an attractive or repulsive response. In addition, these receptors are surprisingly modular; ectodomains and cytoplasmic domains can be swapped without eliminating receptor function. Finally, Hong, Stein, and colleagues (Hong et al., 1999) demonstrate that formation of heteromeric receptor complexes can play a critical role in regulating the response of the growth cone to these complex bifunctional guidance cues.

Cytoplasmic Domains of Guidance Receptors Are Key Effectors, Directing Either Attractive or Repulsive Responses

The *Drosophila* CNS midline expresses both attractants and repellents that are recognized by distinct transmembrane receptors. Roundabout (Robo) is a receptor for the midline repellent signal Slit (Kidd et al., 1998, 1999). Frazzled (Fra), the *Drosophila* DCC ortholog, is the receptor for midline attractants of the Netrin family (Kolodziej et al., 1996 and references therein). The ectodomains of both Robo and Fra encode immunoglobulin domains and fibronectin type III repeats; the cytoplasmic domains of these proteins are unique and do not encode any obvious signal transducing motifs. However, they do encode proline-rich motifs that are evolutionarily conserved and may bind adaptor molecules linking these receptors to signal transduction pathways.

An interesting scenario exists at the midline: CNS midline cells express both attractants and repellents, and many growth cones near the midline simultaneously express both Fra and Robo receptors. If the growth cone is poised to respond to either the attractive or inhibitory signal, what determines the specific response? Bashaw and Goodman (1999) hypothesize that the ectodomains of these receptors determine ligand recognition, while the cytoplasmic domains specify the response. To test this model, they generated chimeric receptors, fusing the ectodomain of Fra with the cytoplasmic domain of Robo (Fra-Robo) and the ectodomain of Robo with the cytoplasmic domain of Fra (Robo-Fra), and then expressed these chimeric receptors in all neurons of the *Drosophila* embryonic CNS (Figure 1A). Bashaw and Goodman observed that these chimeric receptors behaved precisely as predicted by their hypothesis. Neurons expressing high levels of the Fra-Robo chimeric receptor were repelled by Netrin-expressing midline cells leading to a lack of axons crossing the CNS midline. This effect of the Fra-Robo receptor was Netrin dependent. On the other hand, neurons expressing the Robo-Fra chimeric receptor were attracted to the Slit-expressing midline cells, with many axons inappropriately crossing the CNS midline. For both chimeric receptors, a variety of different genetic backgrounds were analyzed to demonstrate that these phenotypes were the result of gain-of-function properties of the chimeric receptor and not simply the result of dominant-negative effects.

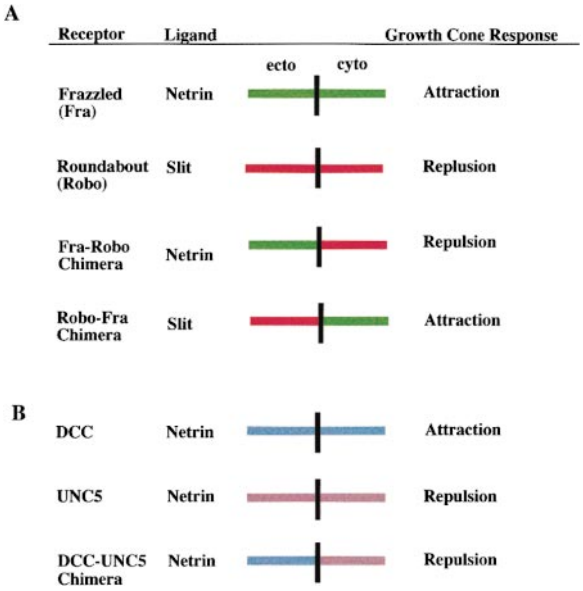

Figure 1. Cytoplasmic Domains of Axon Guidance Receptors Determine Attraction or Repulsion

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What general principles emerge from these in vivo studies? First, it seems that all growth cones posses the appropriate cytoplasmic machinery to respond to either inhibitory or attractive signals. Second, these guidance receptors are unexpectedly modular with the ectodomains determining ligand binding specificity and the cytoplasmic domains encoding effector function. Third, the ability to respond to these axon guidance cues is not restricted to neurons. Bashaw and Goodman found that the migration of muscle precursors normally inhibited by Slit via the Robo receptor can be attracted to Slit when they are expressing the Robo-Fra chimeric receptor. These muscle precursors do not normally express Fra or respond to Netrin cues; however, they clearly can initiate an attractive response under these conditions, indicating that the downstream machinery is present. Finally, phenotypes generated by these chimeric receptors are dependent on the dose or level of expression. This correlation between severity of phenotype and levels of chimeric receptor expression suggests that a balance of attractive versus repulsive signals is being interpreted by these growth cones. Presumably, high expression levels of the chimeric receptors overcome signaling from endogenous receptors leading to altered axon projections.

In independent studies, Hong et al. (1999) focused on attractive and repulsive responses to a single cue, netrin-1, and also found that the cytoplasmic domain of a receptor dictates the growth cone response. These experiments utilized the turning response of isolated *Xenopus* spinal neurons to gradients of netrin-1. In this assay, the attractive turning response of these axons toward netrin-1 requires the DCC receptor, which is normally expressed by these neurons (Figures 1B and 2A; Ming et al., 1997). Candidate receptors for repulsion were provided by UNC5 proteins, a conserved family of netrin-binding proteins that encode an ectodomain with two thrombospondin type 1 repeats and two immunoglobulin domains, and a cytoplasmic domain lacking any clear signaling motifs (i.e., kinase or phosphatase domains; Leung-Hagesteijn et al., 1992; Ackerman et al., 1997; Leonardo et al., 1997). Genetic studies in *C. elegans* indicate that the UNC-5 protein is part of the machinery that recognizes UNC-6, the *C. elegans* netrin ortholog, as a repulsive signal (Hamelin et al., 1993; Colavita and Culotti, 1998, and references therein). Consistent with this, Hong et al. (1999) showed that the attractive response to netrin-1 is converted to a repulsive response when *Xenopus* neurons express an UNC5 receptor; UNC5-expressing axons turn away from the source of netrin-1 protein (Figures 1B and 2B). When a chimeric receptor consisting of a DCC ectodomain and an UNC5 cytoplasmic domain was tested in the axon turning assay, it mediated a repulsive response to the netrin-1 gradient (Figures 1B and 2C). The authors conclude that UNC5 proteins have an evolutionary conserved role in repulsion and that the UNC5 cytoplasmic domain determines the response of growth cones to the netrin-1 signal.

Heteromeric Receptor Complexes Can Regulate the Response to Bifunctional Cues

UNC5 is clearly necessary for recognizing netrin as a repulsive signal, but is it sufficient? Genetic studies from *C. elegans* suggest that UNC-5 alone is not sufficient for mediating repulsion, although it is clearly necessary; these genetic studies indicate that UNC-40, a DCC ortholog, is also required for at least some UNC-6-mediated repulsion (Colavita and Culotti, 1998). To test whether DCC function is also required for UNC5-mediated repulsion in the *Xenopus* axon turning assay, Hong et al. (1999) used a monoclonal antibody directed against the ectodomain of DCC that blocks the netrin-1 attractive turning response. In the presence of this anti-DCC antibody, the repulsive response of UNC5-expressing axons is eliminated (Figure 2D). Under these conditions, axons show no response to the netrin-1 gradient, either positive or negative.

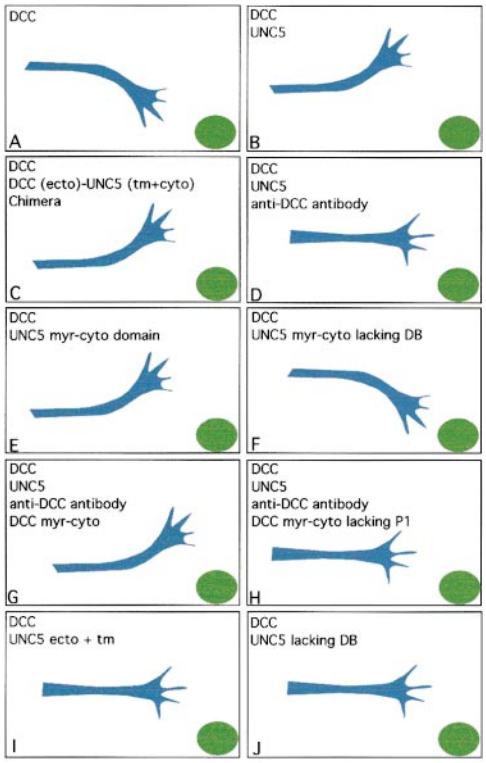

Figure 2. Physiological Evidence that Interactions between DCC and UNC5 Receptors and Their Cytoplasmic Domains Convert Attraction to Repulsion

Schematic diagram of *Xenopus* spinal axons (blue) in vitro reacting to a netrin-1 gradient (green). ecto, ectodomain; tm, transmembrane domain; myr-cyto, myristoylated cytoplasmic domain; DB, required for DCC-binding domain.

Do UNC5 and DCC interact directly to form a complex that mediates netrin-1 repulsion? To test for potential interactions, coimmunoprecipation experiments were conducted with COS cells expressing full-length DCC and UNC5. Association between these two receptors was found, but only in the presence of netrin-1, suggesting that netrin-1 triggers the formation of a UNC5–DCC complex (Figure 3A). This same netrin-dependent association was seen for DCC and UNC5 receptors encoding only ecto- and transmembrane domains (Figure 3B). Interestingly, if the DCC and UNC5 cytoplasmic domains are expressed in COS cells and targeted to the inner membrane with a myristoylation motif, a netrin-independent association is found (Figure 3C). Thus, the

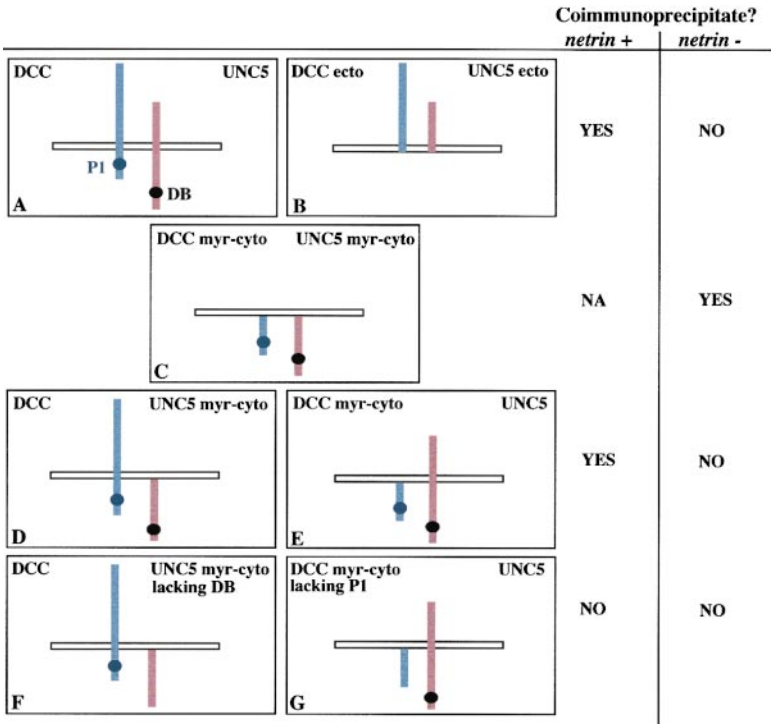

Figure 3. Biochemical Evidence that Netrin-1 Drives Formation of a Heteromeric DCC/UNC5 Receptor Complex and Derepresses an Interaction between Their Cytoplasmic Domains

Schematic diagrams of various forms of DCC (blue) and UNC5 (purple) receptors expressed in COS cells (membrane represented by horizontal rectangle). Abbreviations are as defined in Figure 2.

cytoplasmic domains of DCC and UNC5 can interact; however, this interaction is suppressed in the context of the intact receptors. Surprisingly, binding of netrin-1 to the ectodomain of only one receptor, either DCC or UNC5, is also able to induce association of the cytoplasmic domains (Figures 3D and 3E). These findings suggest a model in which binding of netrin-1 by either of these receptors allows formation of this heteromeric receptor complex and simultaneously derepresses the interaction of their cytoplasmic domains.

But is the cytoplasmic domain interaction important to convert attraction to repulsion? This was tested by first identifying specific sequences necessary for this interaction in both the UNC5 and DCC cytoplasmic domains. A region of DCC just proximal to the transmembrane domain, named the P1 domain, is required for interaction with UNC5 and is conserved among members of the DCC family. Similarly, a region of the UNC5 cytoplasmic domain was identified that was necessary for mediating interactions with DCC. This domain, named DB (required for *D*CC *b*inding), is located in the middle of the cytoplasmic domain and is conserved within the UNC5 family. If either of these domains is deleted, formation of receptor complexes is blocked, even in the presence of netrin-1 (compare Figures 3F and 3G with 3D and 3E).

To test whether these interaction domains, P1 and DB, are required for UNC5-DCC-mediated repulsion, Hong and colleagues returned to the *Xenopus* spinal axon turning assay. Neurons expressing a myristoylated UNC5 cytoplasmic domain and full-length DCC respond to netrin-1 as a repulsive signal (Figure 2E). However, if the DB domain is deleted from this UNC5 cytoplasmic domain, these axons respond to netrin-1 as an attractant, a response mediated by DCC (Figure 2F). To determine the role of the DCC cytoplasmic domain for netrin-1-mediated repulsion, Hong and colleagues blocked endogenous DCC function with the anti-DCC antibody and added a myristoylated DCC cytoplasmic domain. When this DCC cytoplasmic domain is present, UNC5 can mediate a repulsive response to netrin-1 (compare Figure 3D with Figure 3G). This effect is dependent upon interaction of the DCC and UNC5 cytoplasmic domains, since deletion of the DCC P1 domain eliminates this response (Figure 3H). These experiments indicate that netrin-1 binding to the UNC5 ectodomain is sufficient to induce signaling as long as the DCC cytoplasmic domain is present and able to interact with the UNC5 cytoplasmic domain. Clearly, the DCC cytoplasmic domain is essential for UNC5-mediated repulsive signaling as well.

Hong and colleagues suggest that UNC5 acts as a switch in converting an axon's response to netrin-1 from attraction to repulsion. UNC5 receptors usurp DCC receptors when forming heteromeric repulsive receptor complexes. Consistent with this model, two classes of mutant UNC5 receptors actually interfere with endogenous DCC-mediated attractive responses. Axons expressing UNC5 receptors missing either the cytoplasmic domain or simply the DB domain block the response of DCC-expressing neurons to the netrin-1 gradient (Figures 2I and 2J).

What's Next?

Growth cones navigate along their pathways with remarkable speed and fidelity. Clearly a complex set of attractive and repulsive cues are present in the extracellular environment and are being interpreted by the growth cone. These papers demonstrate the critical role that receptors and receptor complexes play as effectors for these different guidance cues. As is often the case,

while questions have been answered, new ones have been raised. Is growth cone guidance a continual battle between these different signals, or a balance between attraction and repulsion, as suggested by Bashaw and Goodman? Is the growth cone a democracy where the majority rules and every signal has an equal voice? Or have systems evolved that allow some signals to dominate at particular times, mechanisms that can switch a growth cone from an attractive to repulsive response rapidly, as is suggested by Hong and colleagues? Most likely, we will find that each of these scenarios is correct and that axon guidance in vivo utilizes both mechanisms.

The next challenge will be to decipher what lies between the cytoplasmic domains of these axon guidance receptors and the cytoskeleton. What are the proteins that bind to these different cytoplasmic domains, and how are they regulated? Bashaw and Goodman point out that several families of repulsive guidance receptors have been identified and yet their cytoplasmic domains share no motifs in common. Does this suggest that these various classes of receptors will utilize distinct signaling pathways? Are there real differences between a Robo-mediated repulsive signal versus an UNC5-DCC repulsive signal? At some point all of these signaling pathways must converge on the proteins that directly regulate assembly and disassembly of the growth cone cytoskeleton. Unraveling how all of this is orchestrated will certainly be fascinating.

Some initial insights are already being generated with the *Xenopus* axon turning assay. Using different pharmacological inhibitors, Poo, Tessier-Lavigne, and colleagues have identified signaling pathways that are required for axon turning to gradients of various attractants and repellents (Ming et al., 1997, 1999; Song et al., 1998). Cyclic nucleotide signaling pathways (both cAMP and cGMP) have dramatic effects on attractive and repulsive turning responses to distinct guidance cues. Phospholipase C-γ, phosphoinoitide 3-kinase, and Ca^{2+} also can play critical roles. These in vitro studies have begun to identify common signaling pathways that are utilized by different classes of guidance cues. We are now poised to ask how these signaling pathways are linked with specific receptors in vivo during the complex process of axon guidance.

Selected Reading

Ackerman, S.L., Kozak, L.P., Przyborski, S.A., Rund, L.A., Boyer, B.B., and Knowles, B.B. (1997). Nature *386*, 838–842.

Bashaw, G.J., and Goodman, C.S. (1999). Cell *97*, this issue, 917–926.

Colavita, A., and Culotti, J.G. (1998). Dev. Biol. *194*, 72–85.

Hamelin, M., Zhou, Y., Su, M.W., Scott, I.M., and Culotti, J.G. (1993). Nature *364*, 327–330.

Hong, K., Hinck, L., Nishiyama, M., Poo, M.M., Tessier-Lavigne, M., and Stein, E. (1999). Cell *97*, this issue, 927–941.

Kidd, T., Brose, K., Mitchell, K.J., Fetter, R.D., Tessier-Lavigne, M., and Goodman, C.S. (1998). Cell *92*, 205–215.

Kidd, T., Bland, K.S., and Goodman, C.S. (1999). Cell *96*, 785–794.

Kolodziej, P.A., Timpe, L.C., Mitchell, K.J., Fried, S.R., Goodman, C.S., Jan, L.Y., and Jan, Y.N. (1996). Cell *87*, 197–204.

Leonardo, E.D., Hinck, L., Masu, M., Keino-Masu, K., Ackerman, S.L., and Tessier-Lavigne, M. (1997). Nature *386*, 833–838.

Leung-Hagesteijn, C., Spence, A.M., Stern, B.D., Zhou, Y., Su, M.W., Hedgecock, E.M., and Culotti, J.G. (1992). Cell *71*, 289–299.

Ming, G.L., Song, H.J., Berninger, B., Holt, C.E., Tessier-Lavigne, M., and Poo, M.M. (1997). Neuron *19*, 1225–1235.

Ming, G.L., Song, H.J., Berninger, B., Inagaki, N., Tessier-Lavigne, M., and Poo, M.M. (1999). Neuron *23*, 139–148.

Song, H.J., Ming, G.L., He, Z., Lehman, M., McKerracher, L., Tessier-Lavigne, M., and Poo, M.M. (1998). Science *281*, 1515–1518.

Tessier-Lavigne, M., and Goodman, C.S. (1996). Science *274*, 1123–1133.